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The impact of a 48-hour fast on SIRT1 and GCN5 in human skeletal muscle

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ABSTRACT

The present study examined the impact of a 48 hour fast on the expression and activation status of SIRT1 and GCN5, and the relationship between SIRT1/GCN5 and the gene expression of PGC-1α, and the PGC-1α target PDK4, in the skeletal muscle of ten lean healthy men (age, 22.0 ± 1.5 years; VO₂peak, 47.2 ± 6.7 mL/min/kg). Muscle biopsies and blood samples were collected 1 hour postprandial (Fed) and following 48 hours of fasting (Fasted). Plasma insulin (Fed, 80.8 ± 47.9 pmol/L; Fasted, not detected) and glucose (Fed, 4.36 ± 0.86; Fasted, 3.74 ± 0.25 mmol/L, \( p = 0.08 \)) decreased, confirming participant adherence to fasting. Gene expression of PGC-1α decreased \( (p < 0.05, -24\%) \), while SIRT1 and PDK4 increased \( (p < 0.05, +11\% \) and \( +1023\%, \) respectively), while GCN5 remained unchanged. No changes were observed for whole muscle protein expression of SIRT1, GCN5, PGC-1α, or COX IV. Phosphorylation of SIRT1, AMPKα, ACC, p38 MAPK, and PKA substrates, as well as nuclear acetylation status was also unaltered. Additionally, nuclear SIRT1 activity, GCN5, and PGC-1α content remained unchanged. Preliminary findings derived from regression analysis demonstrate that changes in nuclear GCN5 and SIRT1 activity/phosphorylation may contribute to the control of PGC-1α, but not PDK4, mRNA expression following fasting. Collectively, and in contrast to previous animal studies, our data are inconsistent with the altered activation status of SIRT1 and GCN5 in response to 48 hours of fasting in human skeletal muscle.

Keywords: Fasting, GCN5, KAT2A, PGC-1α, SIRT1, Skeletal Muscle
INTRODUCTION

Cellular metabolic homeostasis is largely controlled at the transcriptional level by peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α) (Jeninga et al. 2010), a nuclear cofactor proposed to coordinate the activation of both nuclear and mitochondrial transcription of mitochondrial genes (Lin et al. 2002, Scarpulla et al. 2012). While the regulation of PGC-1α is complex, acetylation status has emerged as a key regulator of PGC-1α activity (Jeninga et al. 2010). In cellular and animal models, silent mating type information regulation 2 homologue 1 (SIRT1) deacetylates and activates PGC-1α in response to cellular energy stress (Nemoto et al. 2005, Rodgers et al. 2005, Gerhart-Hines et al. 2007). Conversely, general control non-repressible 5 (GCN5, also known as KAT2A) appears to be the primary acetyltransferase responsible for acetylating and inhibiting PGC-1α (Lerin et al. 2006, Gerhart-Hines et al. 2007). In human skeletal muscle, the role of SIRT1 in controlling PGC-1α activity remains controversial (Gurd 2011, Philp and Schenk 2013), while the importance of GCN5 has not yet been examined. At present, the regulation of SIRT1 and GCN5 expression and activation in response to metabolic stress in human skeletal muscle remains largely unexplored.

In cellular and animal models, skeletal muscle SIRT1 and GCN5 are known to respond to changes in nutrient availability (Coste et al. 2008, Kanfi et al. 2008, Caton et al. 2011, Noriega et al. 2011). As a result, early animal work in skeletal muscle with SIRT1 and GCN5 utilized fasting to examine the regulation of PGC-1α acetylation status (Gerhart-Hines et al. 2007, Coste et al. 2008, Cantó et al. 2010). These studies demonstrated that fasting induces deacetylation and activation of PGC-1α (Gerhart-Hines et al. 2007, Coste et al. 2008, Cantó et al. 2010) subsequent to increases in NAD⁺ (SIRT1 substrate) (Gerhart-Hines et al. 2007, Cantó et al. 2010), and potentially post-translational activation of SIRT1 (Nasrin et al. 2009, Gerhart-Hines et al. 2011).
The activation of SIRT1 by fasting is thought to occur concurrently with decreases in GCN5 activity in mouse skeletal muscle (Coste et al. 2008), which is proposed to be regulated by nuclear translocation and the availability of acetyl-CoA (Jeninga et al. 2010, Philp et al. 2011). Furthermore, fasting increases the expression of SIRT1 and decreases the expression of GCN5 in mouse skeletal muscle (Coste et al. 2008, Kanfi et al. 2008, Noriega et al. 2011), providing an additional potential mechanism by which these factors may regulate the acetylation state of their targets following energetic stress.

Recently, Wijngaarden and colleagues reported that 48 hours of fasting decreases PGC-1α mRNA expression in skeletal muscle of lean healthy men (Wijngaarden et al. 2013). This finding is consistent with an inhibition of PGC-1α activity, as it can autoregulate its own transcription (Handschin et al. 2003). This is in contrast to reports in mice, where PGC-1α mRNA is elevated in response to fasting (Cantó et al. 2010, Caton et al. 2011), supposedly due to an increase in SIRT1 activity and a decrease in GCN5 activity (Gerhart-Hines et al. 2007, Cantó et al. 2010, Jeninga et al. 2010). While these results suggest that the response of SIRT1 and GCN5 to fasting in mice may not be conserved in humans, the impact of fasting on SIRT1 and GCN5 has yet to be examined in human skeletal muscle.

Interestingly, another transcriptional target of PGC-1α, PDK4 (Wende et al. 2005), is reported to have elevated levels of mRNA expression in response to fasting in human skeletal muscle (Spriet et al. 2004, Wijngaarden et al. 2013), a result which is consistent with an increase in PGC-1α activity. Whether changes in SIRT1 and GCN5 expression and/or activity are associated with changes in PGC-1α and/or PDK4 mRNA in human skeletal muscle following fasting is currently unknown. Thus, the purpose of this study was to examine the expression and activation of SIRT1 and GCN5 in human skeletal muscle following a 48-hour fast, and to
examine the relationship between SIRT1 and GCN5 and changes in PGC-1α and PDK4 gene expression.

METHODS

Participants

Ten lean healthy men volunteered to participate in the study (characteristics presented in Table 1). All participants were recreationally active but were not involved in a specific training program at the time of recruitment. Participants were instructed to maintain their regular daily activities, but to avoid exercise and consumption of alcohol or caffeine for 24 hours prior to, and throughout the duration of the fasting period. The experimental protocol and associated risks were explained both orally and in writing to all participants before written consent was obtained. The study was approved by the Health Sciences Research Ethics Board at Queen’s University and conformed to the Declaration of Helsinki. Results from this data set, including participant characteristics and individual changes in PGC-1α gene expression, have been published previously (Walsh et al. 2015).

Experimental Design

Prior to the start of the intervention, the importance of adhering to the fasting protocol was stressed to all participants, and participants were encouraged to withdraw from the study were they unable to fully adhere to the fasting protocol. Participants were provided with dinner (Stouffer’s Sauté Sensations [520 kcal; 74 g carbohydrate (CHO), 10 g fat, 32 g protein], Dole Fruit Crisp [160 kcal; 29 g CHO, 3.5 g fat, 2 g protein], and 500 mL of 2% milk [260 kcal; 24 g CHO, 10 g fat, 18 g protein]) to consume the night before the start of the 48-hour fast, no later than 9:00 pm. The following morning after a 12-hour fast, participants reported to the lab in the fasted state and were fed breakfast consisting of a plain bagel (~190 kcal; 1 g fat, 36 g CHO, 7 g
protein) with peanut butter (110 kcal; 10 g fat, 4 g CHO, 4 g protein) and 200 mL of apple juice (90 kcal; 22 g CHO, 0 g fat, 0 g protein) followed 2 hours later by lunch (12” Black Forest Ham Sandwich from Subway [~690 kcal; 20 g fat, 106 g CHO, 34 g protein] and 500 mL of 2% milk [260 kcal; 24 g CHO, 10 g fat, 18 g protein]). Anthropometric measures (height, weight, and waist circumference) were recorded during this visit.

Venous blood samples were taken 1-hour postprandial (fed state) followed immediately by a muscle biopsy (Edgett et al. 2013). Approximately 24 hours into the fasting period experimenters checked in with participants to monitor their progress and to reinforce the importance of adhering to the fasting protocol. Forty-eight hours post-prandial, participants returned to the lab for a fasted-state blood sample and muscle biopsy (taken from the opposite leg of the fed-state biopsy). During the fasting period participants were provided with six calorie-free electrolyte beverages (Powerade Zero) and were permitted to drink water *ad libitum*. 

**VO₂peak** was measured approximately two weeks following completion of the fasting protocol as described previously (Edgett et al. 2013).

**Blood Analysis**

Plasma samples were collected by venipuncture from an antecubital vein in sterile BD Vacutainer® tubes (BD Diagnostics, Franklin Lakes, NJ, USA) coated with the anticoagulant EDTA. Plasma insulin concentrations were analyzed using a commercially available insulin ELISA kit (80-INSHU-E01.1, ALPCO Diagnostics, Salem, NH, USA) and plasma glucose concentrations were analyzed using a Glucose Colorimetric Assay Kit (#10009582, Cayman Chemicals, Ann Arbor, MI, USA). All blood samples were measured in duplicate and according to the manufacturer's instructions. The average coefficient of variation was 5.4% ± 3.6% and 1.4% ± 1.2% (mean ± SD), for insulin and glucose, respectively.
Preparation of Whole Muscle Lysates

Muscle biopsy samples used to prepare whole muscle lysates were immediately frozen in liquid nitrogen and stored at -80°C until analysis. Approximately 20 mg of frozen wet muscle was homogenized on ice in 500 µL of lysis buffer (210 mM sucrose, 2 mM EGTA, 40 mM NaCl, 30 mM Hepes, 5 mM EDTA), supplemented with protease inhibitors (cOmplete ULTRA Tablets, Mini, EDTA-free, Roche, Mannheim, Germany) and phosphatase inhibitors (PhosSTOP, Roche, Mannheim, Germany), twice for 15 seconds at 20000 RPM (Polytron PT 10-35 GT, Kinematica, Luzern, Switzerland) with a 15 second break in between. The resulting homogenate was then spun at 14000 x g for 30 minutes at 4°C and the supernatant was taken as the whole muscle lysate for western blotting.

Preparation of Isolated Nuclei

Nuclear fractions were prepared from approximately 75 mg of fresh wet muscle using a commercially available kit (NE-PER #78833, Pierce, Rockford, IL, USA) as we and others have done previously (Gurd et al. 2011, Little et al. 2011). Briefly, harvested muscles were immediately placed in 750 µL of PBS, where they were minced and briefly homogenized. Cytoplasmic and nuclear extraction was performed using the cytoplasmic and nuclear extraction reagents supplemented with protease (cOmplete ULTRA Tablets, Mini, EDTA-free, Roche, Basel, Switzerland) and phosphatase inhibitors (PhosSTOP, Roche).

Western Blotting

Nuclear and whole muscle samples were quantified spectrophotometrically to determine protein content (BCA Protein Assay Kit 23225, Pierce, Rockford, IL, USA), then solubilized in sample buffer (12.5% sucrose, 1.9% SDS, 15.6 mM Tris HCl pH 6.8, 0.5 mM EDTA, 0.8% DTT, 0.003% bromophenol blue) and heated to 95°C for 5 minutes. Equal amounts of protein
(12.5-30 µg, depending on the protein of interest) were loaded onto 8-12% polyacrylamide gels and separated by SDS-PAGE. Gels were subsequently transferred to a polyvinylidene difluoride membrane via wet transfer for 1 hour at 100 volts. Membranes were blocked at room temperature by incubating in 5% BSA with TBS-T (0.1%). Blots were then incubated with primary antibodies overnight at 4°C in 5% BSA. Commercially available monoclonal antibodies were used to detect PGC-1α (ST1202; Calbiochem, Darmstadt, Germany); SIRT1 (ab110304; Abcam, Cambridge, MA, USA); GCN5 (#3305), phospho-p38 MAPK Thr180/Tyr182 (#4511), AMPKα (#2532), phospho-AMPKα (Thr172, #2535), and ACC (#3676) (Cell Signaling Technologies, Danvers, MA, USA). Commercially available polyclonal antibodies were used to detect COX IV (#4844), phospho-SIRT1 (Ser47, #2314), acetyl-p53 (Lys382, #2570), Acetylated-Lysine (#9441), phospho-(Ser/Thr) PKA Substrate (#9621), p38 MAPK (#9212), and phospho-ACC (Ser80, #3661; this antibody detects both ACC1 and ACC2; however, ACC2 is the isoform primarily expressed in skeletal muscle, and the corresponding phosphorylation site in humans is Ser222) (Cell Signaling Technologies). Proteins were visualized by chemiluminescent detection according to manufacturer’s instructions (Millipore, Denmark) and imaged using the FluorChem HD2 system (Protein Simple, San Jose, CA). Band intensities were quantified using AlphaView software (Protein Simple). Amido black staining was used as a loading control and did not differ between fed and fasted samples (data not shown).

RNA Extraction and Real-Time PCR

RNA extraction and real-time PCR were performed as we have done previously (Edgett et al. 2013, Scribbans et al. 2014). Briefly, RNA was extracted using a modified version of the single-step method by guanidinium thiocyanate-phenol-chloroform extraction (Chomczyzynski and Sacchi 1987), and then quantified spectrophotometrically at 260 nm using a Take3 Plate.
Protein contamination was assessed by measuring absorbance at 280 nm (samples had an average 260:280 ratio of 1.98 ± 0.02, mean ± SD). One microgram of resulting RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, ON, Canada). Transcript levels were determined on an ABI 7500 Real Time PCR System (Foster City, CA, USA). Primer set efficiencies were determined using real-time PCR with an appropriate cDNA dilution series prior to sample analysis. Average primer set-specific efficiencies (Rasmussen 2001) were $E = 2.03 ± 0.04$ (mean ± SD). All samples were run in duplicate 25 µL reactions containing: 50 ng cDNA, 0.58 µM primers, and GoTaq PCR Master Mix containing SYBR Green (Promega, Madison, WI, USA). All primer sequences are described previously (Edgett et al. 2013), and were designed to amplify across exon–exon boundaries to avoid amplification of genomic DNA. Tata binding protein (TBP) was stable across all states with no difference in the raw $C_T$ values observed between fed and fasted states (Fed 24.18 ± 0.16, Fasted 24.35 ± 0.26, $p = 0.176$). RNA was stored at -80°C and cDNA was stored at -20°C until use.

**Nuclear SIRT1 Activity**

Nuclear SIRT1 activity was measured using a SIRT1 Fluorometric Drug Discovery Kit (BML-AK555, Enzo Life Sciences, Farmingdale, NY, USA) as described by the manufacturer protocols. Briefly, 15 µg of nuclear extract (in 25 µL) was incubated with 15 µL of 325 µM Fluor de Lys®-SIRT1 substrate and 750 µM NAD$^+$ for 30 minutes at 37°C. The reaction was stopped by the addition of 50 µL of developer II reagent and 2 mM nicotinamide, and the fluorescence was subsequently monitored following incubation at room temperature for 60 minutes at 360 nm (excitation) and 460 nm (emission) using a Synergy Mx microplate reader (BioTek). The
specificity of the Fluor de Lys®-SIRT1 substrate has been validated previously (Gurd et al. 2011).

**Immunofluorescent and Histochemical Analysis**

Immunofluorescent analysis of myosin heavy chain isoforms was performed as we have done previously (Bloemberg and Quadrilatero 2012, Scribbans et al. 2014) using primary antibodies against myosin heavy chain (MHC) I (BA-F8), MHCIIa (SCU71), and MHCIIx (6H1) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA). In addition, sections were incubated with a primary antibody against dystrophin [MANDYS1 (3b7), Developmental Studies Hybridoma Bank] to identify the muscle membrane. Fibre types were identified by isotype-specific fluorescent secondary antibodies (type I, blue; type IIA, green; type IIX, red; as well type IIA/IIX hybrid fibres, IIAX). Type IIX and type IIAX fibres were excluded from the current analyses given their relatively low distribution percentage. For all immunofluorescent procedures, sections were mounted with Prolong Gold Antifade Reagent (Life Technologies, Burlington, ON, CA) and imaged the following day. These sections were visualized with an Axio Observer Z1 microscope (Carl Zeiss, Jena, TH, Germany). Individual images were taken across the entire muscle cross-section and assembled into a composite panoramic image using AxioVision software (Carl Zeiss).

Intramuscular triglyceride (IMTG) content was determined with Oil Red O staining (Koopman et al. 2001), whereas glycogen content was determined using the Periodic Acid Schiff (Quadrilatero et al. 2010). Images were acquired with a Brightfield Nikon microscope linked to a PixelLink digital camera. Individual images were taken across the entire muscle cross-section and assembled into a composite panoramic image using Microsoft Image Composite Editor (ICE) (Microsoft, Redmond, WA, USA). Image analysis was performed in ImageJ and calculated by
subtracting background staining. Compiled images were matched to fibre-type images and approximately 40 of each fibre type were randomly selected and analyzed. Data were expressed relative to the values obtained in type I fibres, which were assigned a reference value of 1.0, and reported as mean optical density in arbitrary units (AU).

Statistical Analysis

Statistical analysis of gene expression was performed on linear data using the $2^{\Delta\Delta CT}$ method using TATA-binding protein (TBP) as a reference gene (Schmittgen and Livak 2008). All data was analyzed using a paired Student’s $t$-test with statistical significance set at $p < 0.05$. Simple linear regression was also used to determine the relationships between changes in nuclear PGC-1α, p-SIRT1, SIRT1 activity, and GCN5 with changes in PGC-1α and PDK4 (a downstream target of PGC-1α [Wende et al. 2005]) gene expression. Pearson correlation coefficient ($r$) effect sizes were classified as small ($r = \pm 0.1$), medium ($r = \pm 0.3$), or large ($r = \pm 0.5$) (Field 2013).

RESULTS

Adherence to Fasting Protocol

In addition to verbal confirmation, we examined several measures to confirm participant adherence to the fasting protocol. Consistent with previous reports (Spriet et al. 2004, Wijngaarden et al. 2013), we observed a 10.2 fold increase ($p < 0.0001$) in pyruvate dehydrogenase kinase isozyme 4 (PDK4) mRNA expression in response to prolonged fasting (Fig. 1A). In addition, plasma insulin (Fed, $80.8 \pm 47.9$ pmol/L; Fasted, not detected) and glucose (Fed, $4.36 \pm 0.86$; Fasted, $3.74 \pm 0.25$ mmol/L, $p = 0.0772$) levels decreased (Fig. 1B, C).
Intramuscular Glycogen and Triglyceride Content

There was a significant increase in type I fibre IMTG content in response to fasting (+54%, p < 0.01, Fig. 2A), but not in type IIA fibres. Skeletal muscle glycogen content did not differ in either type I or type IIA fibres between the fed and fasted state (Fig. 2B). Representative images are presented in Figure 2C. Fasting had no effect on fibre-type distribution (data not shown).

SIRT1 & GCN5: Regulators of Nuclear Acetylation Status

In response to 48 hours of fasting, SIRT1 mRNA expression increased 11% (p < 0.01), Fig. 3A); however, there was no associated change in whole muscle protein content (Fig. 3B). In addition, we measured the SIRT1 phosphorylation site at Ser47 because this site is associated with increased SIRT1 nuclear localization and activity (Nasrin et al. 2009). In the present study, whole muscle and nuclear phosphorylated SIRT1 (Ser47) were both unaffected by prolonged fasting (Fig. 4B). Although a 14% decrease in acetylation (Lys382) of the SIRT1 target p53 (p < 0.05, Fig. 4B) was observed, no change in nuclear SIRT1 activity was present (Fig. 3C). GCN5 mRNA expression as well as whole muscle and nuclear protein content were not different in the fasted versus fed state (Fig. 4A, B). In agreement with all of these findings, there was also no change in total nuclear acetylation levels (Fig. 4C).

PGC-1α Expression, Cellular Localization, and Targets

In response to 48 hours of fasting, PGC-1α mRNA expression decreased by 24% (p < 0.0001); however, this was not accompanied by a change in whole muscle protein expression (Fig. 5A, B). PGC-1α is also proposed to be regulated by nuclear translocation (Little et al. 2010, Gurd et al. 2011); however, in the present study, although PGC-1α gene expression decreased, nuclear localization of PGC-1α was not different in the fasted versus fed state (Fig.
5B). Mitochondrial protein cytochrome c oxidase subunit IV (COX IV) protein expression was also unaffected by prolonged fasting (Fig. 5C).

Signalling Events Regulating PGC-1α

The cAMP-dependent protein kinase A (PKA) is also proposed to phosphorylate and activate SIRT1 (Gerhart-Hines et al. 2011); however, at present no commercially available antibody exists for this site in humans. Consequently, we utilized a phospho-PKA substrate-specific antibody to investigate PKA activity. In response to a 48-hour fast, total PKA substrate phosphorylation was unchanged (Fig. 6A). Other signalling kinases proposed to be involved in regulating PGC-1α and SIRT1, such as p38 MAPK (Puigserver et al. 2001) and AMPK (Jäger et al. 2007, Cantó et al. 2009), were also unchanged following 48 hours of fasting (Fig. 6B).

Relationships between Proposed Nuclear Regulators of PGC-1α and PDK4 Gene Expression

We performed simple linear regressions to determine if individual changes in nuclear PGC-1α, p-SIRT1, SIRT1 activity, or GCN5 predicted changes in PGC-1α (Fig. 7) or PDK4 mRNA. While nuclear PGC-1α did not predict PGC-1α gene expression, nuclear GCN5 ($p = 0.034$) accounted for 55% of the variance, and nuclear p-SIRT1 and SIRT1 activity accounted for 44% ($p = 0.051$) and 48% ($p = 0.076$) of the variance, respectively, indicating medium ($r = \pm 0.3$) to large ($r = \pm 0.5$) effect sizes (Field 2013). There was no relationship between any of these variables and PDK4 gene expression (data not shown). Nuclear p-SIRT1 did not predict nuclear SIRT1 activity ($p = 0.101, R^2 = 0.34, r = 0.58$, data not shown).

DISCUSSION

The results of the current study demonstrate that fasting increased SIRT1 mRNA expression, but had no impact on GCN5 mRNA. Additionally, while fasting-induced changes in
PGC-1α and PDK4 gene expression were not explained by sustained changes in SIRT1 activity and/or GCN5 and PGC-1α nuclear translocation at the group level, individual changes in nuclear GCN5 and SIRT1 predicted changes in PGC-1α mRNA, but not PDK4. These results are largely inconsistent with previous animal skeletal muscle models that propose SIRT1 is up-regulated and activated, while GCN5 is down-regulated and inhibited, to promote a net increase in PGC-1α activity in response to fasting (Gerhart-Hines et al. 2007, Coste et al. 2008, Cantó et al. 2010, Caton et al. 2011). Additionally, our observation that muscle glycogen was unchanged following fasting, a finding that contrasts the response in mice (Ryder et al. 1999, Cantó et al. 2010), suggests that fasting does not provide as robust of an energetic stress in human skeletal muscle as it does in murine muscle (Cantó et al. 2010, Caton et al. 2011, Frier et al. 2011). This divergent metabolic response between humans and mice may help explain why changes in PGC-1α gene expression and the regulators of PGC-1α (SIRT1, GCN5, etc.) observed in previous mouse studies (Cantó et al. 2010, Caton et al. 2011) do not seem to occur in human skeletal muscle in response to fasting.

Fasting Alters SIRT1 Gene Expression but Not Activity in Human Skeletal Muscle

In mice, fasting results in either no change (Kanfi et al. 2008, Caton et al. 2011) or an increase in skeletal muscle SIRT1 mRNA expression (Nemoto et al. 2004, Noriega et al. 2011); findings that are somewhat consistent with our observation that SIRT1 mRNA expression is increased in human skeletal muscle following a 48-hour fast. While the mechanism(s) underlying this observed increase in SIRT1 gene expression are unclear, our observation that whole muscle p53 acetylation decreased suggests that p53 may have been inhibited (Brooks and Gu 2011), and that either activation of p53 is not required for fasting-mediated increases in SIRT1 gene expression in humans as it is in mice (Nemoto et al. 2004, Kanfi et al. 2008), or that changes in
whole muscle p53 acetylation do not reflect nuclear p53 activity (Philp and Schenk 2013).
Alternatively, other regulators of SIRT1 gene expression, such as E2F transcription factor 1
(Wang et al. 2006), hypermethylated in cancer 1 (Van Rechem et al. 2010), and early growth
response protein 1 (Pardo et al. 2011) may underlie the observed increase in SIRT1 gene
expression following fasting.

While SIRT1 gene expression was increased following fasting, the lack of associated
changes in SIRT1 protein content or nuclear SIRT1 activity at the group level suggest that
sustained changes in SIRT1 activity do not contribute to the metabolic response of human
skeletal muscle to fasting. These findings are somewhat surprising given the robust effect of
altered nutrient availability on SIRT1 (Nemoto et al. 2004, Rodgers et al. 2005, Coste et al. 2008,
2011) in cellular and animal models. A limitation of the present study is that we were unable to
measure nuclear NAD$^+$ levels, and thus 
in vivo 
SIRT1 activity may have been altered.
Regardless, our results still suggest that while SIRT1 is responsive to fasting in cellular and
animal models, and transient changes in SIRT1 expression/activity in response to shorter-term
fasts may occur, prolonged changes in SIRT1 activity and protein content do not appear to occur
in response to 48 hours of fasting in human skeletal muscle.

*GCN5 Nuclear Localization and Expression is Unaltered Following Fasting in Human Skeletal
Muscle*

Whether GCN5 is involved in regulating mitochondrial biogenesis via PGC-1α in human
skeletal muscle has yet to be explored, and the current study is the first to examine GCN5 protein
content and cellular localization in human skeletal muscle. Surprisingly, although GCN5
expression is altered in response to fasting and high-fat feeding in murine models (Coste et al.
2008, Caton et al. 2011), we observed no change in GCN5 gene expression or protein content in response to a 48-hour fast. Further, we failed to observed any change in nuclear GCN5 protein content, a proposed regulator of GCN5 activity (Philp et al. 2011). Although we observed no change in GCN5 expression or nuclear translocation in the present study, it is possible we may have missed transient changes in GCN5 cellular localization prior to the 48-hour fasted time point. Conversely, GCN5 activity may have been altered via another mechanism, such as acetyl CoA availability (Jeninga et al. 2010), that was not measured in this study. Importantly, our findings do not preclude the involvement of GCN5 in the metabolic response to energetic stress, or the regulation of PGC-1α activity, to different conditions (e.g. exercise) in human skeletal muscle.

Potential Mechanisms Regulating PGC-1α in Human Skeletal Muscle

In agreement with Wijngaarden and colleagues (2013), we observed a decrease in PGC-1α mRNA without a concomitant change in whole muscle protein following a 48-hour fast. Interestingly, the mRNA expression of the PGC-1α downstream target PDK4 (Wende et al. 2005) was elevated following fasting, raising the possibility that PGC-1α activity may have been elevated in response to fasting in human skeletal muscle. Whether PGC-1α or PDK4 gene expression is a more appropriate marker of PGC-1α transcriptional activity remains unknown; however, the changes in PGC-1α and PDK4 appear to occur in the absence of any prolonged changes in SIRT1 activity or GCN5 nuclear localization, PGC-1α nuclear localization, or the phosphorylation status of AMPK, ACC, or p38 MAPK. In agreement with this, phosphorylation of AMPK and ACC are unchanged in human skeletal muscle following a 48-hour fast (Wijngaarden et al. 2013). Interestingly, subsequent to the collection of the current study, Wijngaarden and colleagues (2014) reported a decrease in p-ACC following 10 and 24 hours of
fasting in human skeletal muscle. This suggests that transient changes in molecular signalling, including potential changes in SIRT1 and GCN5, may not have been captured in the present study due to the timing of our muscle samples.

While changes in the nuclear factors proposed to regulate PGC-1α activity were not associated with changes in PGC-1α or PDK4 gene expression on a group level, simple linear regression revealed that the change in nuclear GCN5, p-SIRT1, and SIRT1 activity all predicted changes in PGC-1α, but not PDK4 gene expression. The finding that individual changes in PGC-1α were predicted by changes in SIRT1/GCN5 suggests that these regulators of PGC-1α may be specifically associated with regulation of the PGC-1α gene. Surprisingly, fasting-induced changes in nuclear GCN5 and p-SIRT1 positively predicted changes in PGC-1α mRNA, while the relationship with nuclear SIRT1 activity was negative. These relationships are somewhat in opposition to the current theory that SIRT1 activates, while GCN5 inhibits, PGC-1α activity (Jeninga et al. 2010), and highlight the likely complexity of the fasting response in humans. While the small sample size and preliminary nature of these results suggest that caution is warranted in their interpretation; these findings underscore the need for further research examining the relationships between SIRT1, GCN5 and PGC-1α in human skeletal muscle.

**Time Course of Skeletal Muscle Substrate Utilization in Response to Fasting**

Prolonged fasting in humans is accompanied by significant reductions in circulating insulin and glucose levels, which begin approximately 10-36 (Stannard et al. 2002, Spriet et al. 2004, Wijngaarden et al. 2013, 2014) and 36-48 (Tsintzas et al. 2006, Hoeks et al. 2010, Browning et al. 2012, Wijngaarden et al. 2013) hours into the fasting period, respectively. In agreement with this, we observed a trend for glucose to decrease following a 48-hour fast, and were unable to detect plasma insulin levels in most participants (n = 8). Reductions in circulating
insulin observed in the transition from the post-absorptive to post-prandial state increase plasma FFA and whole-body fat oxidation to spare glucose for the brain (Castillo et al. 1991, Spriet et al. 2004, Tsintzas et al. 2006, Hoeks et al. 2010, Vendelbo et al. 2012). The increase in circulating FFA is proposed to cause elevations in IMTG stores; however, the accumulation is gradual and requires at least 48-hours of fasting (Stannard et al. 2002, Wietek et al. 2004, Hoeks et al. 2010, Browning et al. 2012, Vendelbo et al. 2012), or longer (Browning et al. 2012). Similarly, we observed a significant increase in IMTG content in type 1 fibres following a 48 hour fast. Further, we observed no changes in skeletal muscle glycogen content, which appears to be in agreement with previous reports that human skeletal muscle glycogen remains unchanged following 24 hours of fasting (Maughan & Williams. 1982), and increases in response to 72 hours of fasting (Vendelbo et al. 2012). In contrast, reports in murine fasting models indicate that muscle glycogen can be depleted as early as 12 hours into the fasting period (Ryder et al. 1999, Kokubun et al. 2009, Cantó et al. 2010). Although a limitation of the present study is that participants were not monitored continuously throughout the fasting period, the changes in substrate storage we observed suggest the presence of a similar skeletal muscle metabolic response to fasting as previously reported in humans (Hoeks et al. 2010, Browning et al. 2012, Vendelbo et al. 2012), and also supports the adherence of our participants to the fasting protocol. These findings suggest that that a 48-hour fast does not appear to be the same physiological stress in humans as in murine models, and may partly explain why PGC-1α and its regulators (e.g. AMPK and SIRT1) were largely unchanged in the present study, in contrast to previous animal studies.
Conclusion and Future Directions

This is the first study to investigate SIRT1 and GCN5 expression/activity in human skeletal muscle following a prolonged fast. Our results demonstrate that 48 hours of fasting increased SIRT1 gene expression, but had no effect on nuclear SIRT1 activity, or GCN5 gene expression or nuclear protein content. Interestingly, regression analysis revealed a relationship between the changes in nuclear GCN5 and SIRT1 activity/phosphorylation and PGC-1α, but not PDK4, mRNA expression. While we did observed increases in PDK4 and decreases in PGC-1α gene expression, these changes occurred without any prolonged changes in protein content, or molecular signalling events associated with regulating PGC-1α activity. Although it is possible that fasting may alter PGC-1α and PDK4 expression by mechanisms not examined within the current study, it is also possible that transient changes in SIRT1/GCN5/AMPK occurred, but were missed due to the timing of our post-fasting biopsy (48 hours). Alternatively, unlike in animal and cellular models, the SIRT1/GCN5/PGC-1α axis may not be involved in the fasting response in human skeletal muscle. Unfortunately, we were unable to examine the time course of the present study; however, this remains an interesting topic for future research. While we were also unable to measure nuclear PGC-1α acetylation in the present study, a recent report measuring PGC-1α acetylation in whole muscle human lysates (Seyssel et al. 2014) suggests that nuclear measures may be possible. The development of an IP protocol to determine nuclear PGC-1α acetylation in humans is a critical future research direction in order to fully understand the regulation of PGC-1α activity by SIRT1 and GCN5 in humans. Altogether, the results of the current study suggest further investigation of the fasting response could provide novel insight into the regulation of SIRT1, GCN5, and PGC-1α in human skeletal muscle.
DISCLOSURES

The authors have no conflicts of interest to declare.
REFERENCES


**Table 1. Participant Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Height (cm)</td>
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<tr>
<td>Weight (kg)</td>
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<td>BMI (kg/m)</td>
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<tr>
<td>Waist Circumference (cm)</td>
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</tr>
<tr>
<td>Absolute VO\textsubscript{2}peak (mL/min)</td>
<td>3563 ± 430</td>
</tr>
<tr>
<td>Relative VO\textsubscript{2}peak (mL/min/kg)</td>
<td>46.9 ± 6.0</td>
</tr>
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Values are mean ± standard deviation ($n = 10$)
Figure 1. Impact of a 48-hour fast on metabolic parameters to confirm the effects of fasting. Skeletal muscle biopsies were analyzed for (A) mRNA expression of PDK4 ($n = 9$), while venous blood samples were analyzed for (B) glucose and (C) insulin levels ($n = 10$). Values presented as mean ± SEM. ND = not detectable, where 8 out of 10 participants were below the limit of detection. † $p = 0.0772$, *** $p < 0.0001$ in the fasted vs. fed state.

Figure 2. Effects of a 48-hour fast on skeletal muscle (A) IMTG content, (B) glycogen content, (C) and representative fibre type slides for A and B ($n = 9$). Type I fibres are blue, type IIA fibres are green, and the muscle membrane is red. Values presented as mean ± SEM. Scale bars represent 100 microns (µm). * $p < 0.01$ in the fasted vs. fed state.

Figure 3. Effect of a 48-hour fast on skeletal muscle (A) SIRT1 mRNA expression, (B) total and phosphorylated (Ser47) SIRT1 and acetyl-p53 protein content, (C) nuclear SIRT1 activity, and (D) representative blots for B ($n = 9$). Values presented as mean ± SEM. Whole muscle samples were measured in duplicate and nuclear samples in singlicate for all analyses. †† $p < 0.05$, * $p < 0.01$ in the fasted vs. fed state.

Figure 4. Effect of a 48-hour fast on skeletal muscle (A) GCN5 mRNA expression, (B) whole muscle and nuclear GCN5 protein content, (C) total nuclear acetylated lysine, and (D) representative blot for B and C ($n = 9$; except for nuclear GCN5, $n = 8$). Values presented as mean ± SEM. Whole muscle samples were measured in duplicate and nuclear samples in singlicate for all analyses.
**Figure 5.** Effect of a 48-hour fast on skeletal muscle (A) mRNA expression of PGC-1α, (B) whole muscle and nuclear PGC-1α protein content, (C) whole muscle COX IV protein content, and (D) representative blots for B and C (n = 9). Values presented as mean ± SEM. Whole muscle samples were measured in duplicate and nuclear samples in singlicate for all analyses. ***p < 0.0001 in the fasted vs. fed state.

**Figure 6.** Effects of a 48-hour fast on skeletal muscle (A) phosphorylation of PKA substrates, (B) phosphorylation of p38 MAPK, AMPK, and ACC relative to total protein, (C) and representative blots for A and B (n = 9). Values presented as mean ± SEM. Whole muscle samples were measured in duplicate.

**Figure 7.** Relationship between proposed regulators of PGC-1α gene expression in response to a 48-hour fast in human skeletal muscle. Linear regression plots of (A) nuclear PGC-1α protein content, (B) nuclear GCN5 protein content, (C) nuclear SIRT1 activity, and (D) nuclear p-SIRT1 content with PGC-1α gene expression. Numbers on plots correspond to individual participants.
(A) mRNA Expression (Fold Change vs. Fed State)

(B) Blood Insulin (pmol L\(^{-1}\))

(C) Blood Glucose (mmol L\(^{-1}\))
(A) mRNA Expression (Fold Change vs. Fed State)

(B) Protein Content (Fold Change vs. Fed State)

(C) Arbitrary Fluorescence Units (Fold Change vs. Fed State)

(D) Nuclear SIRT1 Activity

Nuclear Amido Black
Nuclear p-SIRT1 (Ser^47)
Amido Black
SIRT1
p-SIRT1 (Ser^47)
acetyl-p53 (Lys^382)

Fed | Fasted

~ 120 kDa
~ 120 kDa
~ 53 kDa
Below 25 kDa

Amido Black

p-p38 MAPK/
p38 MAPK
p-AMPKα/
AMPKα
p-ACC/
ACC

p-A CC/
A MPK
p-A MAPK/
A MPK

phos-PKA Substrates

Protein Content
(Fold Change vs. Fed State)

phos-PKA Substrates
(Fold Change vs. Fed State)

(A) (B) (C)